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14. ABSTRACT
Mucin1 (MUC1), a glycoprotein is aberrantly overexpressed in TNBC and facilitates growth and metastasis of triple negative breast cancer (TNBC) cells. This occurrence can be partially attributed to MUC1 interaction with hypoxia-inducible factor alpha (HIF1α), a key regulator of glycolysis. We previously observed that ectopic overexpression of MUC1 increased glucose uptake, lactate secretion and enhanced the expression of glycolytic enzymes. Therefore we hypothesized that MUC1 stabilizes HIF1α to facilitate metabolic reprogramming. In the present study MUC1-overexpressing cells (MDA-MB-231.MUC1) demonstrated that MUC1 alters expression of several metabolic genes. Metabolic gene alterations was also observed in MUC1 compared to Neo after stimulating cells with EGF, which induces nuclear localization and transcriptional activation of the cytoplasmic tail of MUC1. Additionally, MUC1 enhanced glutamine uptake that was increased/decreased under hypoxic conditions and increased nucleotide biosynthesis to support cell growth. Lastly, MUC1 increased/decreased oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), indicative of cells utilizing glycolysis and/or oxidative phosphorylation to meet energy requirements. Thus our results support the notion that MUC1 serves as a metabolic regulator in TNBC, facilitating metabolic reprogramming that influences growth of TNBC.

15. SUBJECT TERMS
Cancer metabolism, glycolysis, mucin1, pentose phosphate pathway, triple negative breast cancer

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INTRODUCTION

Breast cancer, the second leading cause of cancer deaths in women, is the most common cancer among North American women, accounting for nearly 1 in 3 cancer cases diagnosed in the U.S. women [1, 2]. Triple negative breast cancer (TNBC) subtype accounts for approximately 15-25% of all breast cancer cases and has an increased incidence of metastasis, high recurrence within 1-3 years and a high mortality rate [3]. Therefore, identifying factors that facilitate tumor growth and metastases have the potential to serve as novel molecular targets for breast cancer therapy. MUC1, a glycoprotein associated with chemoresistance, is aberrantly overexpressed in over 90% of early TNBC lesions [4-6]. Much of the oncogenic potential role of MUC1 can be attributed to the participation of the small, cytoplasmic tail of MUC1 (MUC1 CT) in signal transduction and transcriptional events, facilitating growth and metastasis[6-9]. Oncogenic potential can also be attributed to MUC1 ability to interact and stabilizes hypoxia-inducible factor alpha (HIF1α), a key regulator of glycolysis [10]. As metastasis is the leading cause of cancer related deaths, this process relies on cooperation between the tumor cells and their surrounding stromal, establishing a reactive tumor microenvironment. Stromal cells can serve as a sink for the end-products of aerobic glycolysis (i.e, lactate) and provide a source of metabolites (i.e., pyruvate) to support to support cancer growth, invasion and metastasis [9]. Hence our overall research focus is to investigate how signaling through MUC1 faciliates hypoxia-dependent and independent metabolic cross-talk between epithelial and stromal components in TNBC; thus facilitating tumor growth and metastasis. Additionally, we will examine if co-targeting MUC1 and HIF1α will block epithelial-stroma metabolic cross-talk, diminish chemoresistance and reduce tumor growth and metastasis in TNBC. Findings from the proposed study may identify MUC1 as a novel therapeutic target for breast cancer, particularly for the TNBC subtype.

KEYWORDS: cancer metabolism, glycolysis, mucin1, pentose phosphate pathway, triple negative breast cancer

OVERALL PROJECT SUMMARY

Specific Aim 1

Research Objective: To elucidate the mechanism by which MUC1 modulates tumor-stromal metabolic cross-talk, promoting tumor growth and metastasis in distinct triple-negative breast cancer microenvironment.

Methodology

Cell Culture

MDA-MB231 Neo (Neo) and MDA-MB231 MUC1 (MUC1) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in a humidified atmosphere at 37^{o}C with 5% CO_2 under atmospheric oxygen condition (20%).

Hypoxia To generate hypoxic conditions, the oxygen concentration was set to 1% and the cells were maintained under hypoxia for 24 hours.

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qPCR) was performed in 384 Well Optical Reaction Plates (Applied Biosystems) using SYBRGreen PCR Master Mix (Roche). Reactions were carried out on an ABI 7500 thermocycler (Applied Biosystems). All samples were amplified

in duplicate and quantification of the expression level of each gene was calculated using the delta delta CT method, normalized to β-actin. Data presented as fold change relative to the control (MDA-MB231 Neo).

Glucose Uptake Assay

$5x10^4$ cells were seeded per well in a 24-well plate and allowed to adhere. Cells were then serum starved for 24 h and stimulated with EGF at the indicated concentration and time. Next cells were starved for glucose for 2 h and then incubated for 20 min with 1μCi [^{3}H]-2-deoxyglucose and lyzed with 1%SDS. The lysates were counted for [^{3}H] using a scintillation counter. Cells treated with labeled and excess unlabeled glucose were used as controls to set a baseline for nonspecific tritium uptake. The results were normalized to the cell counts for normoxia and hypoxia groups. Data are presented as the mean value of quadruplicate values of glucose uptake normalized with control cells.

Glutamine Uptake Assay

$5x10^4$ cells were seeded per well in a 24-well plate and allowed to adhere overnight. For hypoxic conditions cells were maintained under hypoxia for 12 hours following overnight incubation. Cells were incubated for 2 min with 3μCi [^{3}H]-glutamine then lyzed with 1%SDS. The lysates were counted for [^{3}H] using a scintillation counter. Cells treated with labeled and excess unlabeled glutamine were used as controls to set a baseline for nonspecific tritium uptake. The results were normalized to the cell counts for normoxia and hypoxia groups. Data are presented as the mean value of quadruplicate values of glutamine uptake normalized with control cells.

Lactate Assay

Lactate levels secreted in the media were analyzed by colorimetric using a L-Lactate Assay Kit (Eton Bioscience Inc.) according to manufactures instructions.

Glycolytic Flux Analysis

Assessment of glycolytic function was performed using Seahorse XF24 analyzer (Seahorse Bioscience). The instrument allows the simultaneous quantification of mitochondrial respiration (oxygen consumption rate, OCR) and glycolysis to lactic acid (extracellular acidification rate, ECAR). Assay was performed according to manufactures instructions. In brief, cells were plated at a density of $4x10^4$ into each well of the XF-24 well plates approximately 18 hours before the analysis. Assays were initiated by removing the growth medium and replacing it with unbuffered media followed by the addition of 10mM glucose to asses rate of glycolysis, 2.5μM oligomycin, an ATP synthase inhibitor, to asses glycolytic capacity and 100mM 2DG, a glucose analog, to inhibit glycolysis. The difference between glycolytic capacity and rate of glycolysis define the glycolytic reserve.

Statistical Analysis: Nonparametric Tukey tests were used to compare differences between cell lines. A p-value of 0.05 or less will be deemed significant.

Results

MUC1 regulates metabolic gene expression in TNBC cells.

Preliminary data showed that MUC1 increased glucose uptake, lactate secretion and glycolytic enzymes, therefore to determine if MUC1 regulates expression of glycolytic, TCA cycle and pentose phosphate pathway (PPP) genes quantitative real-time polymerase chain reaction analysis was utilized. Results indicated that several genes in each pathway were altered comparing MDA-MB-231.MUC1 (MUC1) with control MDA-MB-231.Neo (Neo) cells under normoxic and/or hypoxic conditions (Figure 1).

Glycolysis

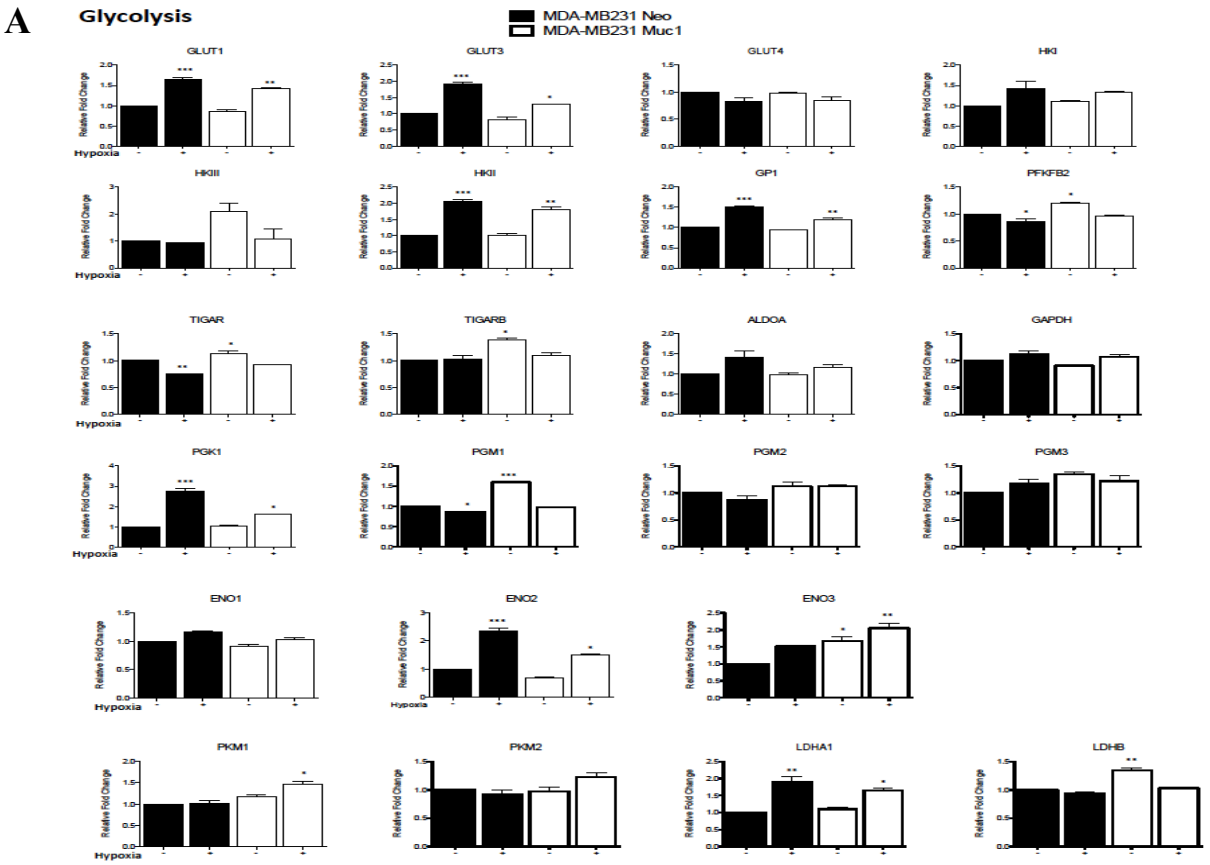
Within the glycolytic pathway hypoxia alone increased the expression of several genes (*GLUT1*, *GLUT3*, *HKII*, *GPI*, *PGK1*, *ENO2*, *LDHA1*, *PDK1*) whose expression was also increased by MUC1 under hypoxic conditions but to a lesser extent. We also observed that MUC1 alone increased the expression of *TIGAR* and *TIGARB* and increased *LDHB* and *PKM1* under hypoxic conditions.

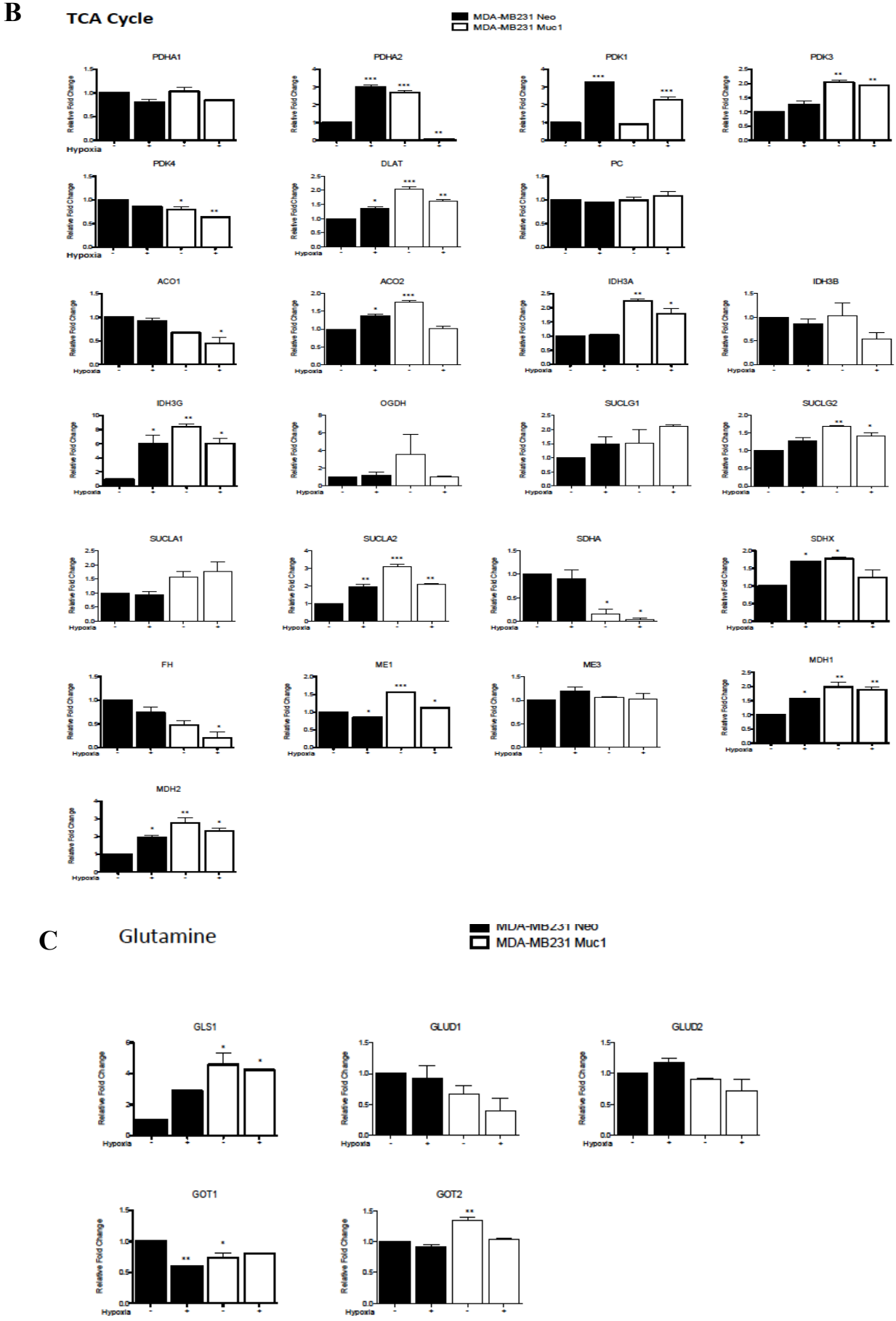
TCA Cycle

The conversion of pyruvate to acetyl CoA is catalyzed by the pyruvate dehydrogenase complex providing a link between glycolysis and the TCA cycle. We observed that hypoxic conditions increased the expression of *DLAT*, which expression was further increased by MUC1 under both normoxic and hypoxic conditions. Hypoxia and MUC1 also increased *PDHA2* expression while MUC1 under hypoxic conditions decreased the expression. Pyruvate dehydrogenase kinases, which inhibit the pyruvate dehydrogenase complex showed increased expression of *PDK1* under hypoxic conditions for both Neo and MUC1 but to a lesser extent in MUC1, increased expression of *PDK3* in MUC1 under normoxic and hypoxic conditions to a similar extent and decreased *PDK4* in MUC1 under normoxic and hypoxic conditions to a similar extent. Within the TCA Cycle hypoxia alone increased the expression of *IDH3G*, *SUCLA2*, *MDH1*, and *MDH2* whose expression was also increased by MUC1 under normoxic and hypoxic conditions. We also observed increased expression of *IDH3A* and *SUCLG2* and decreased expression of *SDHA* in MUC1 under normoxic and hypoxic condition. Lastly, we observed decreased expression of *ME1* under hypoxic condition that was increased in MUC1 under normoxic and hypoxic conditions.

Pentose Phosphate Pathway

The pentose phosphate pathway is required for nucleotide biosynthesis and is the major source for the NADPH required for anabolic processes[11]. Within this pathway MUC1 increased *PGLS* expression and decreased *PGD* expression that was enhanced/diminished under hypoxic conditions respectfully. MUC1 also decreased *RPIA* under hypoxic conditions and increased *TALDO* under normoxic conditions.




B
TCA Cycle
MDA-MB231 Neo
MDA-MB231 Muc1
PDHA1
PDHA2
PDK1
PDK3
PDK4
DLAT
PC
ACO1
ACO2
IDH3A
IDH3B
IDH3G
OGDH
SUCLG1
SUCLG2
SUCLA1
SUCLA2
SDHA
SDHX
FH
ME1
ME3
MDH1
MDH2
Relative Fold Change
Hypoxia
C
Glutamine
MDA-MB231 Neo
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GLS1
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GOT2

D

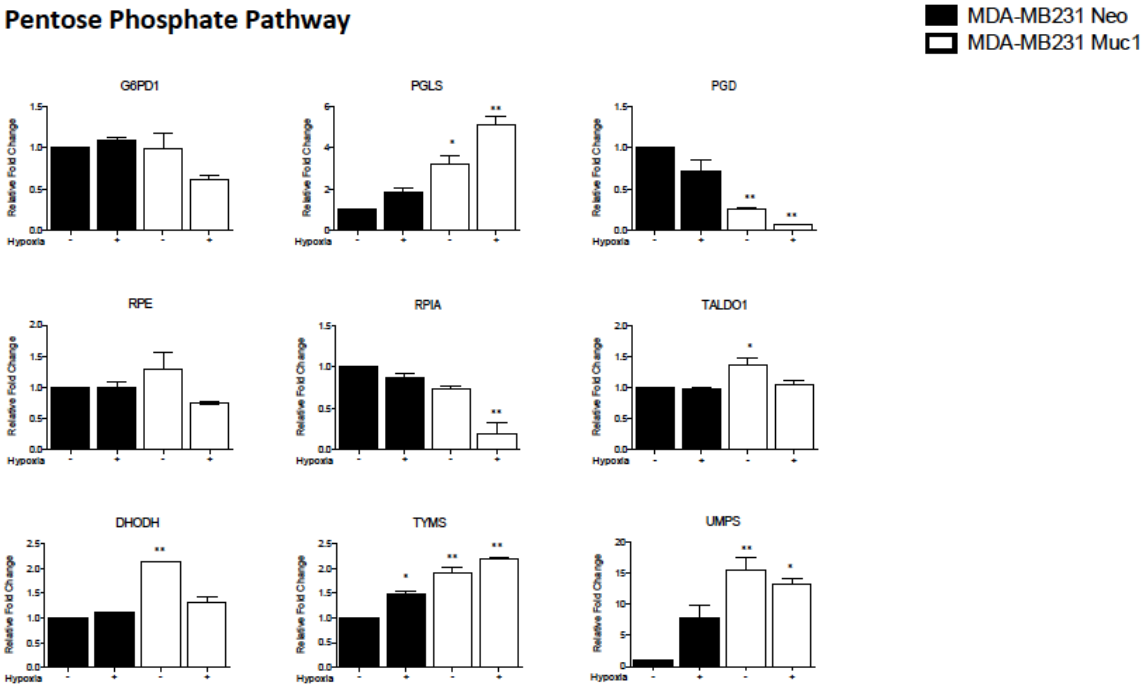


Figure 1. MUC1 regulates metabolic gene expression in TNBC cells. Gene expression analysis of indicated gene under normoxic or hypoxic condtions, comparing MUC1 with Neo. **A.** Glycolysis **B.** TCA Cycle **C.** Glutmaine Uptake **D.** Pentose Phosphate Pathway

MUC1 regulates glutamine uptake in TNBC cells

Glutamine has been shown to play a role in promoting cancer growth, participating in energy formation and redox homeostasis [12]. Therefore glutamine uptake assay was utilized to determine the effect of MUC1 on [^{3}H]-glutamine uptake under normoxic or hypoxic conditions. Results showed a …fold increase in glutamine uptake, suggesting that MUC1 facilitates glutamine uptake (Figure 2). These results indicate that MUC1 facilitates the uptake of glutamine in addition to glucose as a carbon sources.

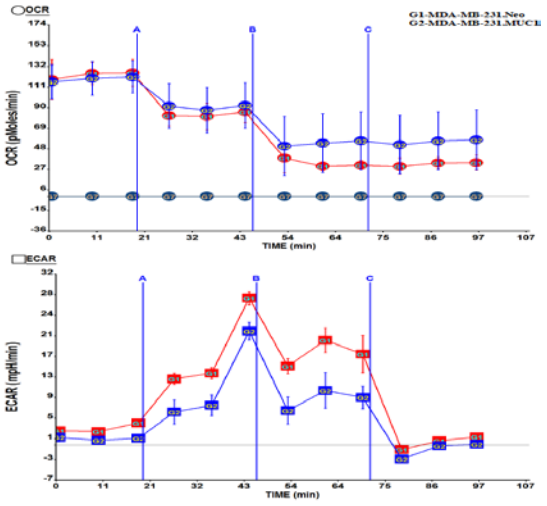


Figure 2. MUC1 regulates glutamate in TNBC cells. 3μCi [^{3}H]-glutamine uptake in Neo and MUC1 cells under normoxic and hypoxic conditions.

MUC1 regulates OCR and ECAR in TNBC cells

To further investigate the differences in metabolic capacity facilitated by MUC1, we analyzed oxygen consumption and extracellular acidification rates by Seahorse XF24 analyzer. We observed no difference in basal OCR or ECAR, a measure of OXPHOS and glycolysis respectfully comparing MUC1 with Neo (Figure 3). Addition of glucose (A) resulted in no change in OCR and decreased in ECAR. Next, addition of oligomycin (B), an ATP synthase inhibitor, increased OCR and decreased ECAR. Lastly, addition of 2DG (C), a glycolysis inhibitor, increased OCR and had no change on ECAR.

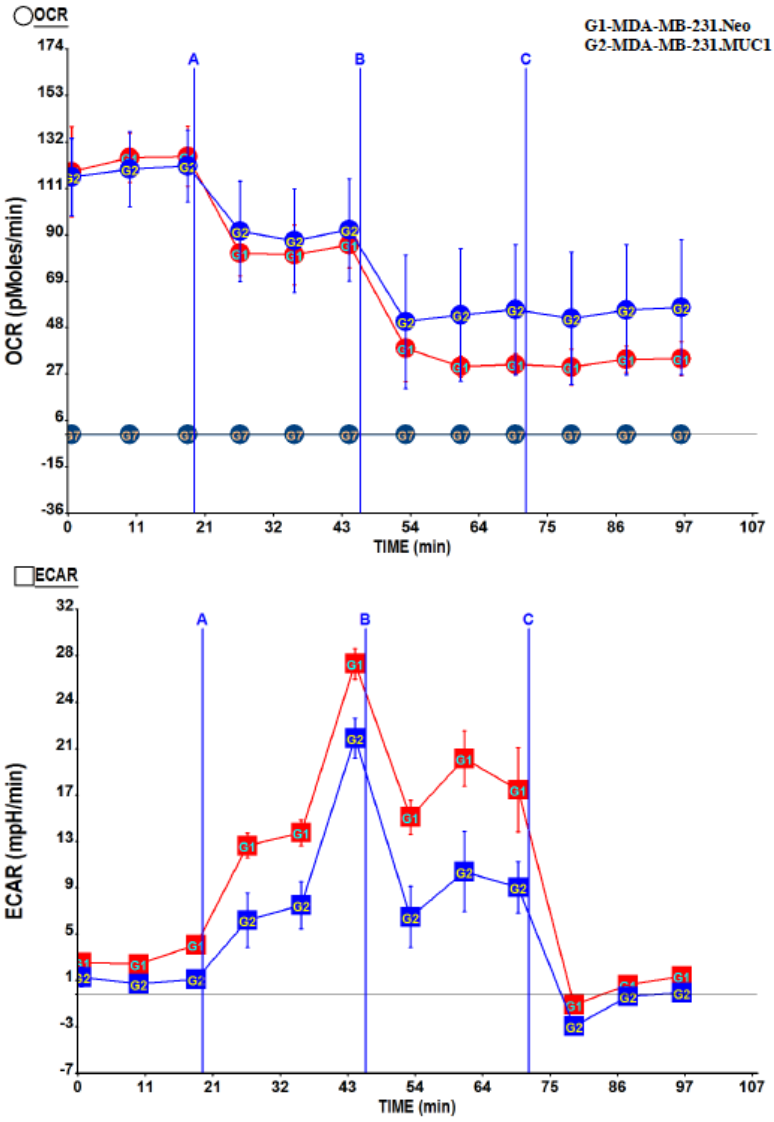


Figure 3. MUC1 regulates OCR and ECAR in TNBC cells. The rate of oxygen consumption and extracellular acidification was determined at basal levels and after the addition of (A) Glucose (B) Oligomycin (C) 2DG

MUC1 regulates EGF stimulated glucose uptake and lactate production in TNBC cells

EGF, which induces nuclear localization and transcriptional activation of the cytoplasmic tail of MUC1, is secreted by stromal cells and has been shown to support tumor growth[8, 9]. Therefore EGF stimulated glucose uptake was assessed in MUC1 compared to Neo in a concentration and time dependent manner. Utilizing 10ng and 100ng concentrations of EGF results indicated that MUC1 facilitates an increase in glucose uptake in a concentration and time dependent manner. showing a …fold increase in glucose uptake (Figure 4). Next, we evaluated EGF stimulated lactate production, an end product of aerobic glycolysis, within the same cell population (Figure 4). EGF stimulation increased lactate production in a concentration and time dependent manner. Additionally, 100ng EGF stimulation induced alteration in several genes regulating glycolysis (Figure 5). EGF stimulation decreased expression of *GLUT1*, *ALDOA* and *LDHA* in MUC1 cells. EGF stimulation also decreased expression of ENO2 and increased PGK1 expression.

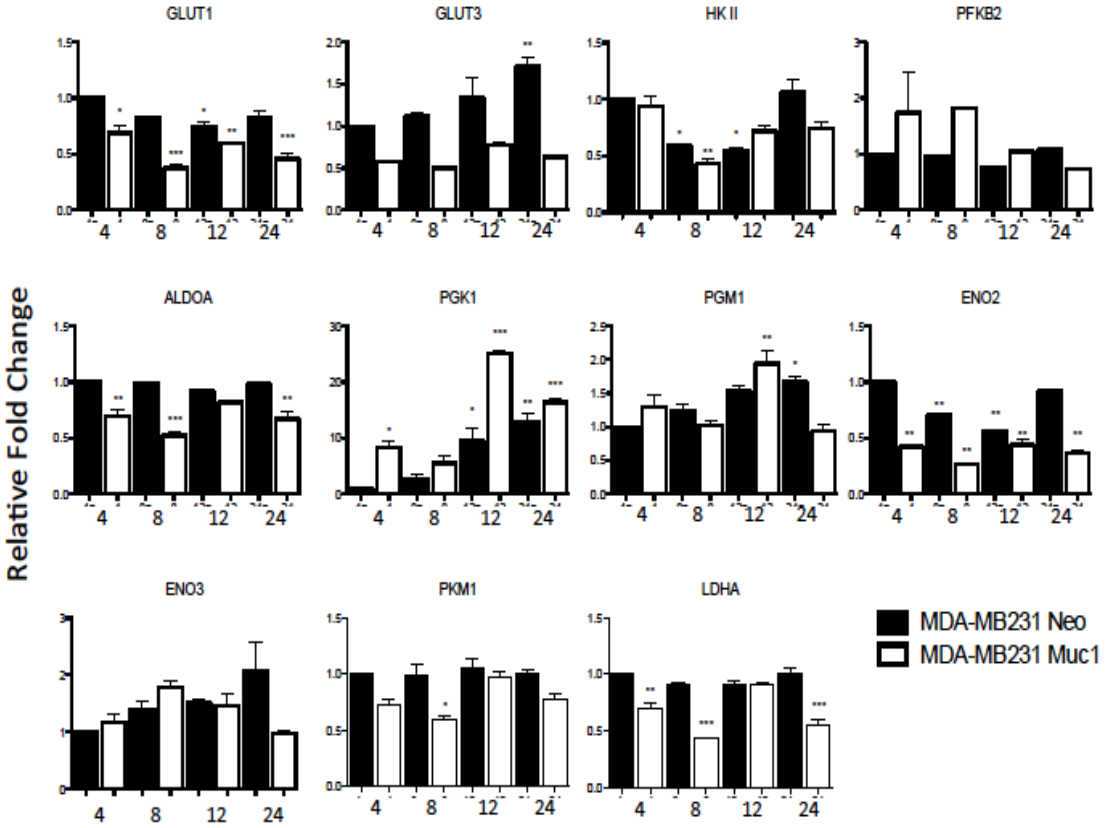
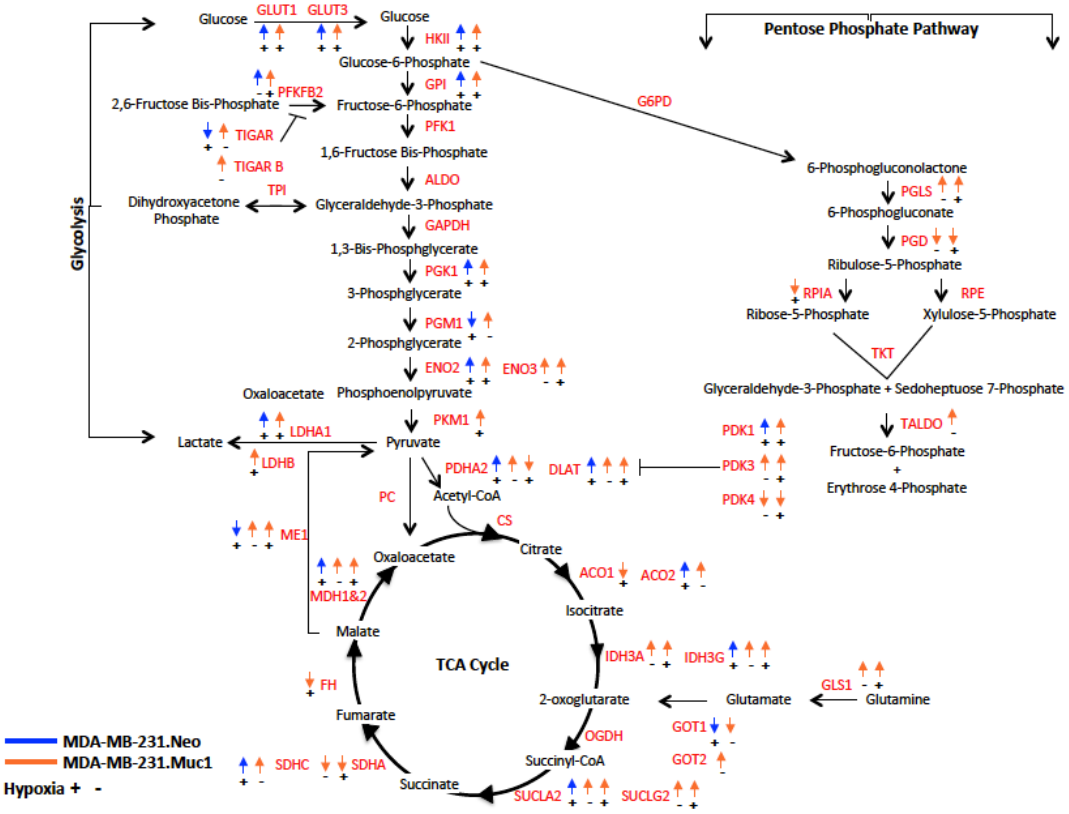


Figure 5. Time course of EGF induced expression of glycolytic genes in TNBC. Expression of indicated gene induced by EGF.

Discussion

In summary, our results support the notion that MUC1 serves as a metabolic regulator in TNBC. Glucose and glutamine serve as the main carbon sources in proliferating cells, and uptake of both nutrients is directed by growth factor signaling[13]. Examining expression of genes regulating metabolic processing of glucose and glutamine revealed MUC1 facilitated alteration in a number of the genes in the absence and presence of hypoxia (Figure 6). This includes genes regulating pyruvate decarboxylation, TCA cycle, oxidative PPP and glutamate conversion from glutamine. In particular, MUC1 facilitated reduction in *PGD* expression suggest blockage of the oxidative branch of the PPP, potentially leading to the accumulation of 6-phosphogluconate and reduction in NAPDH. Consequently in efforts for the cells to maintain NADPH levels they resort to increase glutamine uptake (Figure 2) and glutaminiolysis. Glutaminiolysis, catalyzed by glutaminase (GLS), can lead to the production of malate, a substrate for malic enzyme (ME) to produce pyruvate and NADPH (site). MUC1 facilitated the increase in *GLS* and *ME* supporting this notion (Figure 1). Additionally, the observed decrease in *SDHA* facilitated by MUC1 suggests accumulation of succinate that can ultimately inhibit prolyl hydroxylase (PHD) leading to HIF-1α stabilization and activation[14]. Next, increased OCR following oligomycin treatment suggests increased OXPHOS that can be due to increased expression of *MDH1, MDH2* and *IDH3A*. Furthermore, preciously observed increased glucose uptake (data not shown) and increased glutamine uptake (Figure 2) suggest MUC1 facilitates the utilization of both glucose and glutamine as carbon sources to maximize ATP production.



Metabolic pathway depicting genes regulating glycolysis, PPP, and TCA cycle.

Depiction of genes altered, comparing MUC1 with Neo under normoxic or hypoxic conditions. Blue arrows correspond to genes with decreased/increased expression in Neo cells under hypoxic conditions relative to Neo cell under normoxic conditions. Orange arrows correspond to genes with decreased/increased expression in MUC1 cells under normoxic or hypoxic conditions relative to Neo cells under normoxic conditions.

anticipated problems

We experienced technical difficulties in our MUC1 knockdown experiments. We were unable to package our lentiviral shRNA construct in HEK-293T cells; however after troubleshooting, the issue has been resolved by changing the construct. For our remaining experiments we will utilizes methodologies that are well established in our laboratory and/or the laboratories of collaborators, therefore we do not anticipate further problems.

KEY RESEARCH ACCOMPLISHMENTS

- Determined alterations of key genes regulating metabolic processes facilitated by MUC1
- Determined glutamine uptake is enhanced by MUC1 under normoxic conditions but reduced under hypoxic conditions
- Determined oxygen consumption rate and extracellular acidification rate is by MUC1
- Determined EGF stimulated glucose uptake and lactate production is altered by MUC1 in a concentration and time dependent manner.
- Determined EGF stimulation altered key genes regulating metabolic processes facilitated by MUC1 in a time dependent manner.

CONCLUSION

In conclusion, the data presented here establishes a role of MUC1 in metabolic reprograming in TNBC. Presented data suggest that MUC1 facilitates metabolic reprograming of TNBC to promote cell growth, which can in part be due to the interaction of MUC1 with HIF1α. Our findings also highlight the potential of targeting MUC1 for metastatic breast cancer therapy. To accomplish future goals within the next year we have hired additional personnel and we will utilize techniques previously established in our laboratory. Stable MUC1 knockdown will be established in

additional TNBC cells that exhibit MUC1 overexpression, using shRNA. We will also initiate co-culture with experiments with human mammary fibroblast (hMF) already obtained. Lastly, we will establish stably mCherry expressing hMF and luciferase expressing TNBC cells in order to initiate *in vivo* studies.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press: Nothing to report
(2) Peer-Reviewed Scientific Journals: Nothing to report
(3) Invited Articles: Nothing to report
(4) Abstracts: Nothing to report

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.
Nothing to report

INVENTIONS, PATENTS AND LICENSES: Nothing to report

REPORTABLE OUTCOMES: Nothing to report

OTHER ACHIEVEMENTS: Nothing to report

For each section, 4 through 9, if there is no reportable outcome, state "Nothing to report."

REFERENCES:

1. DeSantis, C., et al., *Breast cancer statistics, 2011.* CA Cancer J Clin, 2011. **61**(6): p. 409-18.
2. Alteri, R., et al., *Breast Cancer Facts & Figures 2011-2012*
, in *Atlanta: American Cancer Society, Inc.*2011, American Cancer Society.
3. Lehmann, B.D., et al., *Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies.* J Clin Invest, 2011. **121**(7): p. 2750-67.
4. Takano, M., et al., *Amplicon profiling reveals cytoplasmic overexpression of MUC1 protein as an indicator of resistance to platinum-based chemotherapy in patients with ovarian cancer.* Oncol Rep, 2004. **12**(6): p. 1177-82.

5. Schroeder, J.A., et al., *MUC1 alters beta-catenin-dependent tumor formation and promotes cellular invasion.* Oncogene, 2003. **22**(9): p. 1324-32.
6. Spicer, A.P., et al., *Delayed mammary tumor progression in Muc-1 null mice.* J Biol Chem, 1995. **270**(50): p. 30093-101.
7. Singh, P.K. and M.A. Hollingsworth, *Cell surface-associated mucins in signal transduction.* Trends Cell Biol, 2006. **16**(9): p. 467-76.
8. Singh, P.K., et al., *Platelet-derived growth factor receptor beta-mediated phosphorylation of MUC1 enhances invasiveness in pancreatic adenocarcinoma cells.* Cancer Res, 2007. **67**(11): p. 5201-10.
9. Behrens, M.E., et al., *The reactive tumor microenvironment: MUC1 signaling directly reprograms transcription of CTGF.* Oncogene, 2010. **29**(42): p. 5667-77.
10. Chaika, N.V., et al., *MUC1 mucin stabilizes and activates hypoxia-inducible factor 1 alpha to regulate metabolism in pancreatic cancer.* Proc Natl Acad Sci U S A, 2012. **109**(34): p. 13787-92.
11. Patra, K.C. and N. Hay, *The pentose phosphate pathway and cancer.* Trends Biochem Sci, 2014. **39**(8): p. 347-54.
12. Wise, D.R. and C.B. Thompson, *Glutamine addiction: a new therapeutic target in cancer.* Trends Biochem Sci, 2010. **35**(8): p. 427-33.
13. Wellen, K.E., et al., *The hexosamine biosynthetic pathway couples growth factor-induced glutamine uptake to glucose metabolism.* Genes Dev, 2010. **24**(24): p. 2784-99.
14. Selak, M.A., et al., *Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase.* Cancer Cell, 2005. **7**(1): p. 77-85.

APPENDICES: none